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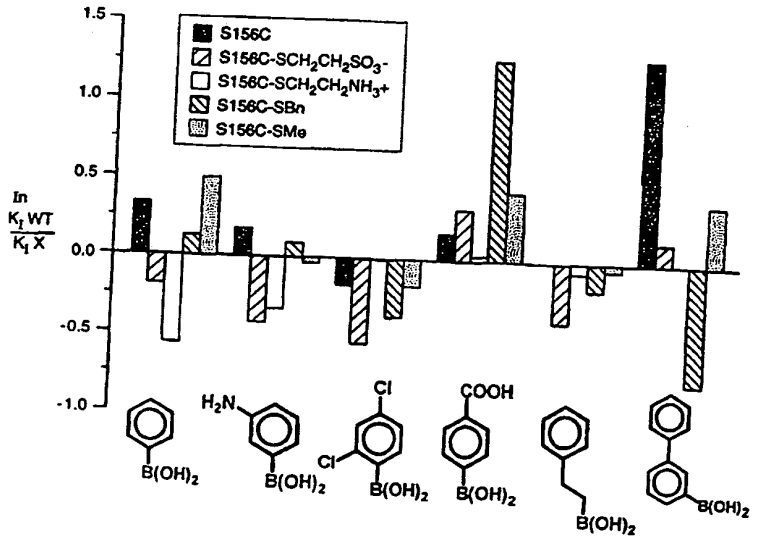

(57) Abstract

Modified enzymes are provided in which at least one amino acid, such as asparagine, leucine, methionine or serine, of an enzyme is replaced with a cysteine and the thiol hydrogen is replaced with a substituent group providing a thiol side chain selected from the group consisting of: a) $-SR^1R^2$, wherein R^1 is an alkyl and R^2 is a charged or polar moiety; b) $-SR^3$, wherein R^3 is a substituted or unsubstituted phenyl; c) $-SR^4$, wherein R^4 is substituted or unsubstituted cyclohexyl; d) $-SR^5$, wherein R^5 is C_{10}–C_{15} alkyl; and e) $-SR^6$ wherein R^6 is a C_{1-6} alkyl. Also, methods of producing the modified enzymes are provided, as well as detergent and feed additives and a composition for the treatment of a textile. A method for using the modified enzymes in organic synthesis is additionally provided. Further, modified enzymes having improved activity, altered pH profile and/or wash performance are provided.

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CHEMICALLY MODIFIED ENZYMES

Background of the Invention

Modifying enzyme properties by site-directed mutagenesis has been limited to natural amino acid replacements, although molecular biological strategies for overcoming this restriction have recently been derived (Cornish, V.W. et al. (1995) Angew. Chem., Int. Ed. Engl. 34:621). However, the latter procedures are not generally easy to apply in most laboratories. In contrast, controlled chemical modification of enzymes offers broad potential for facile and flexible modification of enzyme structure, thereby opening up extensive possibilities for controlled tailoring of enzyme specificity.

Changing enzyme properties by chemical modification has been explored previously, with the first report being in 1966 by the groups of Bender (Polgar, L. et al. (1966) J. Am. Chem. Soc. 88:3153) and Koshland (Neet, K.E. et al. (1966) Proc. Natl. Acad. Sci. USA 56:1606), who created a thiolsubtilisin by chemical transformation ($CH_2OH \rightarrow CH_2SH$) of the active site serine residue of subtilisin BPN' to cysteine. Interest in chemically produced artificial enzymes, including some with synthetic potential, was renewed by Wu, Z.-P. et al. (1989) J. Am. Chem. Soc. 111:4514; Bell, I.M. et al. (1993) Biochemistry 32:3754 and Peterson, E.B. et al. (1995) Biochemistry 34:6616, and more recently by Suckling, C.J. et al. (1993) Bioorg. Med. Chem. Lett. 3:531.

Enzymes are now widely accepted as useful catalysts in organic synthesis. However, natural, wild-type, enzymes can never hope to accept all structures of synthetic chemical interest, nor always to transform them stereospecifically into the desired enantiomerically pure materials needed for synthesis. This potential limitation on the synthetic applicabilities of enzymes has been recognized, and some progress has been made in to altering their specificities in a controlled manner using the site-directed and random mutagenesis techniques of protein engineering. However, modifying enzyme properties by protein engineering is limited to making natural amino acid replacements, and molecular biological methods devised to overcome this restriction are not readily amenable to routine application or large scale synthesis. The generation of new specificities or activities obtained by chemical modification of enzymes has intrigued chemists for many years, and continues to do so. The inventors have adopted the combined site-directed mutagenesis-chemical modification strategy since it offers virtually unlimited possibilities for creating new structural environments at any amino acid location.

US Patent 5,208,158 describes chemically modified detergent enzymes wherein one or more methionines have been mutated into cysteines. The cysteines are subsequently modified in order to confer upon the enzyme improved stability towards

oxidative agents. The claimed chemical modification is the replacement of the thiol hydrogen with a C_{1-6} alkyl.

Although US Patent 5,208,158 has described altering the oxidative stability of an enzyme, it would also be desirable to develop one or more enzymes with altered properties such as activity, nucleophile specificity, substrate specificity, stereoselectivity, thermal stability, pH activity profile and surface binding properties for use in, for example, detergents or organic synthesis.

Summary of the Invention

There exists a need for enzymes such as proteases that have altered properties. As such, the present invention provides modified enzymes that have one or more amino acid residues replaced by cysteine residues. The cysteine residues are modified by replacing the thiol hydrogen with a substituent group providing a thiol side chain selected from the group consisting of :

a) $-SR^1R^2$, wherein R^1 is an alkyl and R^2 is a charged or polar moiety;

b) $-SR^3$, wherein R^3 is a substituted or unsubstituted phenyl;

c) $-SR^4$, wherein R^4 is substituted or unsubstituted cyclohexyl; and

d) $-SR^5$, wherein R^5 is C_{10}-C_{15} alkyl.

In preferred embodiments, the thiol side chain groups $-SR^3$ and $-SR^4$ above, further comprise an alkyl group, R, which is placed before either R^3 or R^4 to form $-SRR^3$ or $-SRR^4$. R is preferably a C_{1-10} alkyl.

With regard to the thiol side chain group $-SR^1R^2$, R^2 can be positively or negatively charged. Preferably, R^2 is SO_3^-, COO^- or NH_3^+. Further, R^1 is preferably a C_{1-10} alkyl.

Preferably, the enzyme is a protease. More preferably, the enzyme is a *Bacillus* subtilisin. Also, preferably, the amino acids therein replaced by cysteines are selected from the group consisting of asparagine, leucine, methionine or serine. More preferably, the amino acid to be replaced is located in a subsite of the protease, preferably, the S_1, S_1' or S_2 subsites. Most preferably, the amino acids to be replaced are N62, L217, M222, S156 and S166 where the numbered position corresponds to naturally-occurring subtilisin from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other subtilisins, such as *Bacillus lentus* subtilisin.

In a particularly preferred embodiment, the enzyme is a *Bacillus lentus* subtilisin. In the most preferred embodiments, the amino acid to be replaced by cysteine is N62 and the thiol side chain group is selected from the group:

$-S^1R^2$ wherein R^1 is CH_2 and R^2 is $CH_2SO_3^-$;

$-SRR^3$ wherein R is CH_2 and R^3 is C_6H_5;

$-SRR^4$ wherein R is CH_2 and R^4 is $c\text{-}C_6H_{11}$;

$-SR^5$ wherein R^5 is $n\text{-}C_{10}H_{21}$; or

the amino acid to be replaced by cysteine is L217 and the thiol side chain group is $-SR^5$ wherein R^5 is $n\text{-}C_{10}H_{21}$.

The present invention further provides modified enzymes that have one or more amino acid residues replaced by cysteine residues. The cysteine residues are modified by replacing the thiol hydrogen with a substituent group providing a thiol side chain $-SR^6$ wherein R^6 is a $C_{1\text{-}6}$ alkyl and the amino acid residues to be replaced by cysteine are selected from the group consisting of asparagine, leucine, and serine. Preferably, the enzyme is a protease. More preferably, the enzyme is a *Bacillus* subtilisin. Most preferably, the amino acid is located in a subsite of the protease, preferably, the S_1, S_1' or S_2 subsites. Most preferably, the amino acids to be replaced are N62, L217, M222, S156 and S166. Preferably, the enzyme is a *B. lentus* subtilisin, the amino acid to be replaced by a cysteine is N62 or L217 and the thiol side chain group is $-SR^6$ wherein R^6 is $CH_2C(CH_3)_3$ or C_5H_{11}.

The present invention provides a method of producing a modified enzyme, including providing an enzyme wherein one or more amino acids have been replaced with cysteine residues and replacing the thiol hydrogen of the cysteine residue with a subtituent group providing a thiol side chain selected from the group consisting of :

a) $-SR^1R^2$, wherein R^1 is an alkyl and R^2 is a charged or polar moiety;

b) $-SR^3$, wherein R^3 is a substituted or unsubstituted phenyl;

c) $-SR^4$, wherein R^4 is substituted or unsubstituted cyclohexyl; and

d) $-SR^5$, wherein R^5 is $C_{10}\text{-}C_{15}$ alkyl.

In preferred embodiments, the thiol side chain groups $-SR^3$ and $-SR^4$ above, further comprise an alkyl group, R, which is placed before either R^3 or R^4 to form $-SRR^3$ or $-SRR^4$. R is preferably a $C_{1\text{-}10}$ alkyl.

With regard to the thiol side chain group $-SR^1R^2$, R^2 can be positively or negatively charged. Preferably, R^2 is SO_3^-, COO^- or NH_3^+. Further, R^1 is preferably a $C_{1\text{-}10}$ alkyl.

Preferably, the enzyme is a protease. More preferably, the enzyme is a *Bacillus* subtilisin. Also, preferably, the amino acids therein replaced by cysteines are selected from the group consisting of asparagine, leucine, methionine or serine. More preferably, the amino acid to be replaced is located in a subsite of the protease, preferably, the S_1, S_1' or S_2 subsites. Most preferably, the amino acids to be replaced are N62, L217, M222, S156 and S166 where the numbered position corresponds to naturally-occurring subtilisin from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other subtilisins, such as *Bacillus lentus* subtilisin.

In a particularly preferred embodiment, the enzyme is a *Bacillus lentus* subtilisin. In the most preferred embodiments, the amino acid to be replaced by cysteine is N62 and the thiol side chain group is selected from the group:

$-S^1R^2$ wherein R^1 is CH_2 and R^2 is $CH_2SO_3^-$;

$-SRR^3$ wherein R is CH_2 and R^3 is C_6H_5;

$-SRR^4$ wherein R is CH_2 and R^4 is c-C_6H_{11};

$-SR^5$ wherein R^5 is n-$C_{10}H_{21}$; or

the amino acid to be replaced by cysteine is L217 and the thiol side chain group is

$-SR^5$ wherein R^5 is n-$C_{10}H_{21}$.

The present invention further provides modified enzymes that have one or more amino acid residues replaced by cysteine residues. The cysteine residues are modified by replacing the thiol hydrogen with a substituent group providing a thiol side chain $-SR^6$ wherein R^6 is a C_{1-6} alkyl and the amino acid residues to be replaced by cysteine are selected from the group consisting of asparagine, leucine, and serine. Preferably, the enzyme is a protease. More preferably, the enzyme is a *Bacillus* subtilisin. Most preferably, the amino acid is located in a subsite of the protease, preferably, the S_1, S_1' or S_2 subsites. Most preferably, the amino acids to be replaced are N62, L217, M222, S156 and S166. Preferably, the enzyme is a *B. lentus* subtilisin, the amino acid to be replaced by a cysteine is N62 or L217 and the thiol side chain group is $-SR^6$ wherein R^6 is $CH_2C(CH_3)_3$ or C_5H_{11}.

There are further provided detergent additives that include modified enzymes.

There are provided feed additives that include modified enzymes.

There is provided methods of using the modified enzymes in a detergent formulation.

There is provided methods of using the modified enzymes in the treatment of fabric.

There is provided methods of using the modified enzymes in the preparation of a feed additive.

There are provided modified enzymes having increased activity.

There are provided modified enzymes having altered pH profiles.

There are provided modified enzymes having improved wash performance.

There are provided methods of using the modified enzymes in organic synthesis.

Brief Description of the Drawings

Figure 1 is a bar graph of the results obtained after probing modified S156C mutants with boronic inhibitors at pH 8.6.

Figure 2 is a bar graph of the results obtained after probing modified S166C mutants with boronic inhibitors at pH 8.6.

Figure 3 is a graph of the pH profiles of wild type *Bacillus lentus* subtilisin (SBL-WT, squares) and a modified N62C mutant (N62C-Scy; circles). Points were done in duplicate.

Detailed Description of the Invention

In one embodiment of the invention, a modified enzyme and a method of providing such are provided that has one or more amino acid residues of a subtilisin replaced by cysteine residues. The cysteine residues are then modified by replacing the thiol hydrogen with a substituent group providing a thiol side chain selected from the group consisting of :

a) $-SR^1R^2$, wherein R^1 is an alkyl and R^2 is a charged or polar moiety;

b) $-SR^3$, wherein R^3 is a substituted or unsubstituted phenyl;

c) $-SR^4$, wherein R^4 is substituted or unsubstituted cyclohexyl; and

d) $-SR^5$, wherein R^5 is C_{10}-C_{15} alkyl.

In preferred embodiments, the thiol side chain groups $-SR^3$ and $-SR^4$ above, further comprise an alkyl group, R, which is placed before either R^3 or R^4 to form $-SRR^3$ or $-SRR^4$. R is preferably a $C_{1\text{-}10}$ alkyl.

With regard to the thiol side chain group $-SR^1R^2$, R^2 can be positively or negatively charged. Preferably, R^2 is SO_3^-, COO^- or NH_3^+. Further, R^1 is preferably a $C_{1\text{-}10}$ alkyl.

Preferably, the enzyme is a protease. More preferably, the enzyme is a *Bacillus* subtilisin. Also, preferably, the amino acids therein replaced by cysteines are selected from the group consisting of asparagine, leucine, methionine or serine. More preferably, the amino acid to be replaced is located in a subsite of the protease, preferably, the S_1, S_1' or S_2 subsites. Most preferably, the amino acids to be replaced are N62, L217, M222, S156 and S166 where the numbered position corresponds to naturally-occurring subtilisin from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other subtilisins, such as *Bacillus lentus* subtilisin.

In a particularly preferred embodiment, the enzyme is a *Bacillus lentus* subtilisin. In the most preferred embodiments, the amino acid to be replaced by cysteine is N62 and the thiol side chain group is selected from the group:

$-S^1R^2$ wherein R^1 is CH_2 and R^2 is $CH_2SO_3^-$;

$-SRR^3$ wherein R is CH_2 and R^3 is C_6H_5;

$-SRR^4$ wherein R is CH_2 and R^4 is c-C_6H_{11};

$-SR^5$ wherein R^5 is n-$C_{10}H_{21}$; or

the amino acid to be replaced by cysteine is L217 and the thiol side chain group is

$-SR^5$ wherein R^5 is n-$C_{10}H_{21}$.

The present invention further provides modified enzymes that have one or more amino acid residues replaced by cysteine residues. The cysteine residues are modified by replacing the thiol hydrogen with a substituent group providing a thiol side chain $-SR^6$ wherein R^6 is a C_{1-6} alkyl and the amino acid residues to be replaced by cysteine are selected from the group consisting of asparagine, leucine, and serine. Preferably, the enzyme is a protease. More preferably, the enzyme is a *Bacillus* subtilisin. Most preferably, the amino acid is located in a subsite of the protease, preferably, the S_1, S_1' or S_2 subsites. Most preferably, the amino acids to be replaced are N62, L217, M222, S156 and S166. Preferably, the enzyme is a *B. lentus* subtilisin, the amino acid to be replaced by a cysteine is N62 or L217 and the thiol side chain group is $-SR^6$ wherein R^6 is $CH_2C(CH_3)_3$ or C_5H_{11}.

A "modified enzyme" is an enzyme that has been changed by replacing an amino acid residue such as asparagine, serine, methionine or leucine with a cysteine residue and then replacing the thiol hydrogen of the cysteine with a substituent group providing a thiol side chain, i.e., a group such as a C_{1-6} alkyl or a C_{10-15} alkyl or a group that includes a phenyl group, a cyclohexyl group or a charged or polar moiety. After modification, the properties of the enzyme, i.e., activity or substrate specificity, may be altered. Preferably, the activity of the enzyme is increased.

The term "enzyme" includes proteins that are capable of catalyzing chemical changes in other substances without being changed themselves. The enzymes can be wild-type enzymes or variant enzymes. Enzymes within the scope of the present invention include pullulanases, proteases, cellulases, amylases and isomerases, lipases, oxidases and reductases. The enzyme can be a wild-type or mutant protease. Wild-type proteases can be isolated from, for example, *Bacillus lentus* or *Bacillus amyloliquefaciens* (also referred to as BPN'). Mutant proteases can be made according to the teachings of, for example, PCT Publication Nos. WO 95/10615 and WO 91/06637.

Several types of moieties can be used to replace the thiol hydrogen of the cysteine residue. These include $-SR^1R^2$, $-SR^3$, $-SR^4$, $-SR^5$ or $-SR^6$. R and R^1 are independently defined as a substituted or unsubstituted C_{1-10} alkyl. R^2 is a charged or polar group. R^3 is a substituted or unsubstituted phenyl group. R^4 is a substituted or unsubstituted cyclohexyl group. R^5 is a C_{10-15} alkyl. R^6 is a C_{1-6} alkyl. R^1, R^5 or R^6 can be substituted or unsubstituted and/or straight chain or branched chain. A charged group is one or more atoms that together form a charged molecule, i.e., SO_3^-, COO^- or NH_3^+.

The terms "thiol side chain group", "substituent group providing a thiol side chain", "thiol containing group", and "thiol side chain" are terms which are can be used interchangeably and include groups that are used to replace the thiol hydrogen of the cysteine used to replace one of the amino acids in a subtilisin. Commonly, the thiol side

chain group includes a sulfur through which the R^x groups defined above are attached to the thiol sulfur of the cysteine.

The term "substituted" refers to a group of which a hydrogen of the group has been replaced with another atom or molecule. For example, a hydrogen can be substituted, for example, with a methyl group, a fluorine atom or a hydroxyl group. In the present invention, the alkyl groups, cyclohexyl group and phenyl group can be substituted, i.e., have substitutions of one or more hydrogen atoms with another atom or molecule.

The binding site of an enzyme consists of a series of subsites across the surface of the enzyme. The substrate residues that correspond to the subsites are labeled P and the subsites are labeled S. By convention, the subsites are labeled S_1, S_2, S_3 ,S_4, S_1' and S_2'. A discussion of subsites can be found in Siezen et. al. (1991) Protein Engineering 4:719-737 and Fersht, A.E. (1985) Enzyme Structure and Mechanism 2 ed., Freeman (New York) pp. 29-30. The preferred subsites are S_1, S_1' and S_2.

The amino acid residues of the present invention can be replaced with cysteine residues using site-directed mutagenesis methods or other methods well known in the art. (See, for example, PCT Publication No. WO 95/10615.) A method of modifying the thiol hydrogen of the cysteine residue can be found in Example 4 below.

In one aspect of the invention, the modified protease has altered proteolytic activity as compared to the precursor protease, since increasing such activity (numerically larger) enables the use of the enzyme to more efficiently act on a target substrate. Also of interest are modified enzymes having altered activity, nucleophile specificity, substrate specificity, stereo selectivity, thermal stability, pH activity profile and surface binding properties as compared to the precursor.

Surprisingly, modified proteases of the present invention can have altered pKas and hence the pH profiles that are shifted from that of the precursor protease (see Example 7) without changing the surface charge of the protease molecule.

Modified enzymes of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about 0.01 to about 5% (preferably 0.1% to 0.5%) by weight. These detergent cleaning compositions or additives can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

Modified enzymes of the invention, especially subtilisins, are useful in formulating various detergent compositions. A number of known compounds are suitable surfactants useful in compositions comprising the modified enzymes of the invention. These include nonionic, anionic, cationic, anionic or zwitterionic detergents, as disclosed in US 4,404,128 to Barry J. Anderson and US 4,261,868 to Jiri Flora et al. A suitable detergent formulation

is that described in Example 7 of US Patent 5,204,015. The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the modified enzymes of the present invention may be used for any purpose that native or wild-type enzymes are used. Thus, these modified enzymes can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide synthesis, feed applications such as feed additives or preparation of feed additives, waste treatment, textile applications such as the treatment of fabrics, as fusion-cleavage enzymes in protein production, etc. The modified enzymes of the present invention may comprise improved wash performance in a detergent composition (as compared to the precursor). As used herein, improved wash performance in a detergent is defined as increasing cleaning of certain enzyme-sensitive stains such as grass or blood, as determined by light reflectance evaluation after a standard wash cycle.

The addition of the modified enzymes of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range and the temperature is below the described modified enzyme's denaturing temperature. In addition, modified enzymes of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

In another aspect of the invention, the modified enzyme is used in the preparation of an animal feed, for example, a cereal-based feed. The cereal can be at least one of wheat, barley, maize, sorghum, rye, oats, triticale and rice. Although the cereal component of a cereal-based feed constitutes a source of protein, it is usually necessary to include sources of supplementary protein in the feed such as those derived from fish-meal, meat-meal or vegetables. Sources of vegetable proteins include at least one of full fat soybeans, rapeseeds, canola, soybean-meal, rapeseed-meal and canola-meal.

The inclusion of a modified enzyme of the present invention in an animal feed can enable the crude protein value and/or digestibility and/or amino acid content and/or digestibility coefficients of the feed to be increased, which permits a reduction in the amounts of alternative protein sources and/or amino acids supplements which had previously been necessary ingredients of animal feeds.

The feed provided by the present invention may also include other enzyme supplements such as one or more of β-glucanase, glucoamylase, mannanase, α-galactosidase, phytase, lipase, α-arabinofuranosidase, xylanase, α-amylase, esterase, oxidase, oxido-reductase and pectinase. It is particularly preferred to include a xylanase as a further enzyme

supplement such as a subtilisin derived from the genus *Bacillus*. Such xylanase are for example described in detail in PCT patent publication WO 97/20920.

One aspect of the invention is a composition for the treatment of a textile that includes MP. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

...e modified enzymes of the present invention can be used in organic synthesis to, for example, catalyze a desired reaction and/or favor a certain stereoselectivity. See, for example, Noritomi et al. Biotech. Bioeng. 51:95-99 (1996); Dabulis et al. Biotech. Bioeng. 41:566-571 (1993); Fitzpatrick et al. J. Am. Chem. Soc. 113:3166-3171 (1991).

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

Experimental

Example 1

Producing the Cys-Mutants

The gene for subtilisin from *B. lentus* (SBL) was cloned into the bacteriophage M13mp19 vector for mutagenesis. (US Patent 5,185,258.) Oligonucleotide-directed mutagenesis was performed as described in Zoller et al. (1983) Methods Enzymol. **100**:468-500. The mutated sequences were cloned, excised and reintroduced into the expression plasmid GG274 in the *B. subtilis* host. PEG (50%) was added as a stabilizer. The crude protein concentrate obtained was purified by first passing through a Sephadex™ G-25 desalting matrix with a pH 5.2 buffer (20 mM sodium acetate, 5 mM $CaCl_2$) to remove small molecular weight contaminants. Pooled fractions for the desalting column were then applied to a strong cation exchange column (SP Sepharose™ FF) in the sodium acetate buffer (above), and SBL was eluted with a one step gradient of 0-200 mM NaCl acetate buffer, pH 5.2. Salt-free enzyme powder was obtained following dialysis of the eluent against Millipore purified water, and subsequent lyophilization. The purity of the mutant and wild-type enzymes, which had been denatured by incubation with 0.1 M HCl at 0°C for 30 minutes, was ascertained by SDS-PAGE on homogeneous gels using the Phast™ System from Pharmacia (Uppsala, Sweden). The concentration of SBL was determined using the Bio-Rad (Hercules, CA) dye reagent kit which is based on the method of Bradford (1976) Analytical Biochemistry **72**:248-254. Specific activity of the enzymes was determined in pH 8.6 buffer using the method described below.

Example 2

Preparation of Certain Moieties

3-methylbutyl methanethiosulfonate

The reaction mixture of 1-bromo-3-methylbutane (1.7520 g, 0.0116 mol) and sodium methanethiosulfonate (1.554 g, 0.0116 mol) in dry DMF (5 mL) was heated at 50°C for 2 hr. At room temperature, water (15 mL) was added and the mixture was extracted with ether (3x30 mL). The combined extracts were washed with brine, dried, concentrated. The residue was subjected to flash column chromatography on silica gel with EtOAc-hexanes (1:4). The product was obtained as a colorless liquid (1.4777 g, 70%). IR (film): 3030 (w), 3011 (w), 2958 (st), 2932 (st), 2873 (st), 1468 (m), 1410 (w), 1388 (w), 1367 (w), 1319 (st), 1136 (st), 955 (st), 748 cm^{-1} (st); ^1H NMR (200 MHz, $CDCl_3$): δ 3.33 (s, 3H, CH_3SO_2S); 3.19 (t, J = 7.1 Hz, 2H, SCH_2CH_2), 1.70-1.58 (m, 3H, $SCH_2CH_2CHMe_2$), 0.95 (d, J = 5.3 Hz, 6H, $CHMe_2$); ^{13}C NMR (50 MHz, $CDCl_3$): δ 50.60, 38.19, 34.59, 27.40, 22.06.

Neopentyl methanethiosulfonate

The reaction mixture of neopentyl iodide (3.054 g, 0.0154 mol), sodium methanethiosulfonate (2.272 g, 0.0170 mol) and dry DMF (4 mL) was heated at 90°C for 90 hr. The reaction flask was wrapped with aluminum foil to avoid direct sunlight to the reaction mixture, since the iodide was sensitive to sunlight. At the end of the heating, the reaction mixture was red-brown in color. At room temperature, water (15 mL) was added and the mixture was extracted with ether (3x30 mL). The combined ether extracts were washed twice with brine, dried, concentrated and the residue was subjected to column chromatography on silica gel with EtOAc-hexanes (1:2) to afford a colorless oil which slowly solidified (1.2395 g, 44%). The product was recrystallized from 95% EtOH. mp: 28.5-29.0°C; IR (CH_2Cl_2 cast): 3021 (m), 2956 (m), 2868 (m), 1467 (m), 1433 (m), 1321 (st), 1310 (st), 1125 (st), 951 (m), 757 (m) and 724 cm^{-1} (m); ^1H NMR (200 MHz, $CDCl_3$): δ 3.32 (s, 3H, CH_3SO_2S), 3.13 (s, 2H, SCH_2C), 1.05 (s, 9H, CMe_3); ^{13}C NMR (50 MHz, $CDCl_3$): δ 50.23, 50.09, 32.14, 28.79, MS (EI): 182 (M^+), 57 (base peak, CMe_3^+).

Hexyl methanethiosulfonate

The reaction mixture of 1-bromohexane (1.046 g, 0.00635 mol), sodium methanethiosulfonate (0.850 g, 0.00635 mol) and dry DMF (6 mL) was heated at 60°C for 2 hr. At room temperature, water (15 mL) was added and the resulting mixture was extracted with ether (3x30 mL). The extracts were washed with brine, dried, concentrated and the residue was subjected to flash column chromatography on silica gel with EtOAc-hexanes (1:4) to afford a colorless liquid (2.057 g, 82%). IR ($CDCl_3$ cast): 3030 (w), 3010 (w), 2955 (st), 2930 (st), 2860 (st), 1460 (m), 1320 (st), 1133 (st), 955 (st), 747 cm^{-1} (st); ^1H

NMR (200 MHz, $CDCl_3$): δ 3.33 (s, 3H, CH_3SO_2S), 3.18 (t, J = 7.4 Hz, 2H, SCH_2CH_2), 1.77 (pseudo p, J = 7.2 Hz, 2H, SCH_2CH_2), 1.50-1.20 (m, 6H, $CH_2CH_2CH_2CH_3$), 0.90 (m, 3H, CH_2CH_3); ^{13}C NMR (50 MHz, $CDCl_3$): δ 50.64, 36.50, 31.13, 29.46, 28.26, 22.44, 13.96

Cyclohexylmethyl methanethiosulfonate

The reaction mixture of bromomethylcyclohexane (1.560 g, 0.00881 mol), sodium methanethiosulfonate (1.180 g, 0.00881 mol) and dry DMF (6 mL) was heated at 50°C for 24 hr. At room temperature, water (15 mL) was added and the mixture was extracted with ether (3x30 mL). The extracts were washed with brine, dried, concentrated and the residue was subjected to flash column chromatography on silica gel with EtOAc-hexanes (1:4) to afford a colorless oil (1.5033 g, 82%). IR ($CDCl_3$ cast): 3030 (w), 3012 (w), 2926 (st), 2853 (st), 1446 (m), 1410 (m), 1320 (st), 1134 (st), 955 (st), 746 cm^{-1} (st); ^1H NMR (200 MHz, $CDCl_3$): δ 3.32 (s, 3H, CH_3SO_2S), 3.07 (d, J = 6.9 Hz, 2H, SCH_2CH), 1.95-1.55 (m, 6H), 1.40-0.90 (m, 5H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 50.42, 43.30, 37.83, 32.43, 26.02, 25.82.

Decyl methanethiosulfonate

The mixture of 1-bromodecane (2.095 g, 0.00947 mol), sodium methanethiosulfonate and dry DMF (6 mL) was heated at 60°C for 2 hr. At room temperature, water (15 mL) was added and the mixture was extracted with ether (3x30 mL). The ether extracts were washed with brine, dried, concentrated and the residue was subjected to flash column chromatography on silica gel with EtOAc-hexanes (1:4) to afford a white solid (2.063 g, 94%). It was recrystallized from 95% EtOH. mp: 28.0-29.5°C. IR ($CDCl_3$ cast): 2954 (m), 2921 (st), 2852 (st), 1469 (m), 1305 (st), 1128 (st), 965 (m), 758 (m) and 720 cm^{-1} (m); ^1H NMR (200 MHz, $CDCl_3$): δ 3.32 (s, 3H, CH_3SO_2S), 3.17 (t, J = 7.4 Hz, 2H, SCH_2CH_2), 1.77 (m, 2H, SCH_2CH_2), 1.50-1.20 (m, 14H, $-(CH_2)_7-$), 0.88 (m, 3H, CH_2CH_3); ^{13}C NMR (50 MHz, $CDCl_3$): δ 50.64, 36.49, 31.84, 29.45 (two carbons), 29.37, 29.23, 28.94, 28.57, 22.64, 14.08.

Sodium methanethiosulfonate

Mesyl chloride (46.6 mL, 0.602 mol) was added dropwise to a solution of $Na_2S{\bullet}9H_2O$ (142.2 g, 0.592 mol) in water (150 mL) at 80°C. After the addition, the reaction mixture was heated under reflux and it turned from pale yellow to yellow in 15 hr. During this time, some yellow precipitates were also formed. The reaction mixture was cooled to room temperature and the water was evaporated. After the solid residue was ground with a mortar and pestle and the powder was dried further at 50°C and 1 torr. Absolute ethanol (700 mL) was used to triturate the powder in 4 portions and the ethanol filtrate was concentrated and cooled with an ice bath to obtain a precipitate which was collected by

vacuum filtration. The filtrate was concentrated further to obtain a second crop of precipitates. After repeated concentration and filtration (4 X), the final volume of the filtrate was approximately 10 mL. The combined precipitates were redissolved in absolute ethanol at room temperature and filtered to remove trace amounts of sodium chloride and sodium sulfide. The filtrate was concentrated and cooled and the solids collected by vacuum filtration. Again, the concentration, cooling and filtration process was repeated 3 times to give white, flaky crystals, which were dried further at 1 torr overnight. (24.51 g, 31%) IR (KBr): 3004, 2916, 1420, 1326, 1203, 1095, 980, 772 cm^{-1}. ^1H NMR (200 MHz, D_2O): δ 3.23 (s). ^{13}C NMR (50 MHz, D_2O, with DMSO-d_6 as an internal standard): δ 39.72 ppm.

Benzyl methanethiosulfonate

Benzyl bromide (9.07 g, 0.053 mol) was slowly added to a suspension of sodium methanethiosulfonate (7.10 g, 0.0530 mol) in absolute EtOH (100 mL) and the reaction mixture was heated at reflux overnight. The reaction mixture was cooled with an ice bath and the solid (sodium bromide and sodium methanethiosulfonate) was filtered off. The filtrate was concentrated to give a crude product which was mainly the desired product. Pure product was obtained by flash chromatography on silica gel with EtOAc-hexanes (1:6) (7.92 g, 74%). The product was further purified by recrystallization from absolute ethanol. mp 39.5-40.2°C (lit. 40-42.5°C) IR (KBr): 3089, 3068, 3017, 3000, 2981, 2936, 2918, 1602, 1582, 1496, 1305, 1131, 960, 771, 741, 702 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 7.38 (m, 5H, phenyl), 4.38 (s, 2H, SCH_2), 2.91 (s, 3H, CH_3SO_2). ^{13}C NMR (50 MHz, $CDCl_3$): δ 135.12, 129.14, 129.03, 128.26, 51.09, 40.79.

The reagents CH_3SO_2-$SCH_2CH_2SO_3^-Na^+$ and CH_3SO_2-$SCH_2CH_2NH_3^+Br^-$ were purchased from Toronto Research Chemicals (Toronto, Ontario).

Example 3

Modification of the Cys-Mutants

The following is exemplary for the method used to modify the Cys-mutants, i.e., N62C.

Modification of M222C

To a solution of the Cys-mutant, M222C, *B. lentus* (25.1 mg, 0.94μmol) in buffer (250 ml; 70 mM CHES, 5 mM MES, 2 mM $CaCl_2$, pH 9.5) in a polypropylene test tube which had been precoated with a water solution of polyethylene glycol 10,000 (0.1% w/v), was added a solution of methyl methanethiosulfonate described in Example 2 in 95% EtOH (100 μl, 92.4 μmol). The solution was vortexed and allowed to slowly rotate on an end-over-end rotator at room temperature (22°C). One blank containing ethanol instead of the

reagent-solution was run in parallel. The modification was followed by activity measurements on 10 µl withdrawn samples and was determined according to the method described above. The reaction was terminated after 2.5 hours when addition of another aliquot of reagent to the reaction did not change the activity of the protease. The solution (2.5 ml) was purified on a disposable desalting column (Pharmacia Biotech PD-10™, Sephadex™ G-25M). The column was equilibrated with buffer (25 ml; 5 mM MES, 2 mM $CaCl_2$, pH 6.5) and the sample was loaded on top. The initial 2.5 ml collected was discarded. Protein was eluted with MES-buffer (3.5 ml) and collected in three fractions. All fractions appeared as one single band when checked on gel (SDS-PAGE, Pharmacia Phast-System™) and could not be differentiated from the Cys-mutant or the wild-type which both were run as references. The three fractions were mixed and dialyzed against deionized water (3 x 1 l) at 0°C, followed by lyophilization overnight which gave the modified mutant (14.3 mg). The specific activity was 64.3 U/mg as compared with the Cys mutant (47.1 U/mg).

Measuring the Activity of the Modified Proteases

Activity, including the kinetic parameters k_{cat}, K_M, and k_{cat}/K_M were measured for hydrolysis of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide using the method described in Bonneau, P. et al. (1991) J. Am. Chem. Soc., **113**(3):1030. Briefly, a small aliquot of subtilisin variant stock solution was added to a 1 cm cuvette containing substrate dissolved in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.5 M NaCl and 1% DMSO, and thermostated at 25°C or similarly at pH 8.6, 0.1 M tris buffer containing 0.05% Tween™80 and 1% DMSO. The reaction progress was followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm using a Perkin Elmer λ2 spectrophotometer ($\Delta\varepsilon_{410}$ 8800 $M^{-1}\cdot cm^{-1}$). Kinetic parameters were obtained by measuring initial rates at substrate concentrations of 0.25 mM-4.0 mM (eight concentrations) and fitting this data to the Michaelis-Menten equation.

Table 1 shows the abbreviations for certain of the thiosulfonates. Table 2 shows the kinetic parameters of the modified *B. lentus* subtilisins (SBL) and the precursor subtilisin (SBL-WT) at pH 7.5. The modified enzymes were prepared as described above after site-directed mutagenesis to replace the amino acid of interest with a cysteine. The kinetic parameters were determined at pH 7.5 as described above. The precursor protease was a *Bacillus lentus* subtilisin (SBL-WT).

Table 1

Abbreviation	Structure
-SBn	$-SCH_2C_6H_5$
-S*iso*-butyl	$-SCH_2CH(CH_3)_2$
-S*neo*-pentyl	$-SCH_2C(CH_3)_3$
$-SCH_2$cyclohexyl	$-SCH_2$-c-C_6H_{11}
-Sdecyl	-S-n-$C_{10}H_{21}$

Table 2

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M
SBL-WT	0.55	48	87
N62C	1.49	61	41
N62C-$SCH_2CH_2NH_3^+$	1.2	63	52
N62C-$SCH_2CH_2SO_3^-$	0.83	66	86
N62C-S*iso*-butyl	0.84	76	90
N62C-SBn	0.37	70	189
N62C-S*neo*-pentyl	0.78	96	123
N62C-S-hexyl	0.54	136	252
N62C-SCH_2cyclohexyl	0.48	135	281
N62C-Sdecyl	0.35	69	197
L217C	0.9	16.1	18
L217C-$SCH_2CH_2NH_3^+$	0.71	12.4	17
L217C-$SCH_2CH_2SO_3^-$	0.77	20.6	27
L217C-S*iso*-butyl	0.53	37	70
L217C-SBn	0.65	31.6	49
L217C-S*neo*-pentyl	0.47	40	85
L217C-Shexyl	0.45	61	136
L217C-SCH_2cyclohexyl	0.51	29.8	58
L217C-Sdecyl	0.55	77	140
M222C	0.77	17.3	22
M222C-$SCH_2CH_2NH_3^+$	0.61	1.06	1.7
M222C-$SCH_2CH_2SO_3^-$	0.55	1.64	3
M22C-SBn	0.67	6.9	10
S156C	0.65	43	66
S156C-$SCH_2CH_2NH_3^+$	0.86	39	45
S156C-$SCH_2CH_2SO_3^-$	0.78	31.6	40
S156C-S*iso*-butyl	0.60	24.2	40
S156C-SBn	0.54	21.8	40
S166C	0.51	14.2	28
S166C-$SCH_2CH_2NH_3^+$	0.60	16.3	27
S166C-$SCH_2CH_2SO_3^-$	0.70	3.8	5.4
S166C-S*iso*-butyl	0.91	29	32
S166C-SBn	0.74	6.9	9

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Example 4

Altering the Specificity of the *B. lentus* Subtilisin

Changes in substrate specificity, particularly the S_1 subsite specificity, can be shown by using various boronic acids as competitive inhibitors. Four of the modified S156C mutants and three of the modified S166C mutants described above were evaluated using boronic acid inhibitors. The modified mutants were S156C-SMe, S156C-SBn, S156C-$SCH_2CH_2SO_3^-$, S156C-$SCH_2CH_2NH_3^+$, S166C-$SCH_2CH_2SO_3^-$, S166C-$SCH_2CH_2NH_3^+$, and S166C-SBn.

The boronic acids were prepared, and their inhibition constants measured at pH 8.6 (Waley (1982) Biochem. J. **205**:631-33), as previously described in Seufer-Wasserthal et al. (1994) Bioorganic and Medicinal Chemistry **2**:35-48). The results are shown in Figures 1 and 2.

Example 5

Wash Performance Test

The wash performance of several of the modified enzymes described in the previous examples was evaluated by measuring the removal of stain from EMPA 116 (blood/milk/carbon black on cotton) cloth swatches (Testfabrics, Inc., Middlesex, NJ 07030) which had been pre-bleached in the following manner: in a 4-liter glass beaker, 1.9 grams perborate tetrahydrate, 1.4 grams perborate monohydrate and 1 gram TAED (tetraacetylethylenediamine) were dissolved in 3 liters of deionized water at 60°C for 1 minute with stirring. 36 EMPA 116 swatches were added and stirred for 3 minutes. The swatches were immediately rinsed with cold deionized water for 10 minutes. Swatches were laid flat on absorbent paper towels to dry overnight.

Five pre-bleached EMPA 116 swatches were placed in each pot of a Model 7243S Tergotometer (United States Testing Co., Inc., Hoboken, NJ) containing 1000 ml of water, 3 gpg hardness (Ca^{++}:Mg^{++}::3:1::w:w), 0.67 g of detergent with bleach and enzyme as appropriate. The detergent base was WFK1 detergent from *wfk* - Testgewebe GmbH, Adlerstrasse 42, Postfach 13 07 62, D-47759 Krefeld, Germany.

Detergent Base Component	% of Final Formulation
Zeolite A	25%
Sodium sulfate	25%
Soda ash	10%
Linear alkylbenzenesulfonate	8.8%
Alcohol ethoxylate (7-8 EO)	4.5%
Sodium soap	3%
Sodium silicate (SiO_2:Na_2O::3.3:1)	3%

To this base detergent, the following additions were made:

Bleach Component	% of Final Formulation
Sodium perborate monohydrate	7%
Sodium perborate tetrahydrate	9.2%
TAED	4.5%

Sodium perborate monohydrate and sodium perborate tetrahydrate were obtained from Degussa Corporation, Ridgefield Park, NJ 07660. TAED (tetraacetylethylenediamine) was obtained from Warwick International, Limited, Mostyn, Holywell, Clwyd CH8 9HE, England.

The pre-bleached EMPA 116 swatches were washed in detergent with 0.1 ppm enzyme for 20 minutes at 20°C and were subsequently rinsed twice for 5 minutes in 1000 ml water. Swatches were dried and pressed, and the reflectance from the swatches measured using the L value on the lab scale of a Minolta Chroma Meter, Model CR-200 (Minolta Corporation, Ramsey, NJ 07446). Performance is reported as percent stain removal and percent stain removal relative to native *B. lentus* protease. Percent stain removal was calculated using the equation:

$$\frac{\text{(L value washed swatches) - (L value unwashed swatches)}}{\text{(L value unstained EMPA 221 swatches) - (L value unwashed swatches)}} \times 100$$

Table 3

Enzyme	Percent Stain Removal	Percent Relative Stain Removal
SBL-WT	8.1	100
N62C-$SCH_2CH_2SO_3^-$	13.4	165
S166C-$SCH_2CH_2SO_3^-$	12.8	158
L217C-$SCH_2CH_2SO_3^-$	13.2	163

Example 7

Altering the pH Profile of a Precursor Subtilisin

To examine the effects of chemical modification on the pH profile of SBL, seven modified N62C mutants were made as described above. 0.02M ethylene diamine buffers of ionic strength 0.05M (adjusted with KCl) were employed with 1.25×10^{-4} M succinyl-AAPF-pNA substrate and K_{cat}/K_M measurements were performed as described above. K_{cat}/K_M reflects the pKa of His64, part of the catalytic triad for SBL, in the free enzyme and is unaffected by nonproductive binding modes. Fersht, A.E. (1985) Enzyme Structure and Mechanism 2 ed., Freeman (New York). pKa was calculated using GraphIt (McGeary & Associates, Middletown CT). The shift in pKa reflects a shift in the pH profile of SBL.

Representative pH profiles for SBL N62C-Scylcohexyl (N62C-Scy) and SBL-WT are shown in Figure 3 ([E]=1x10-7 to 5x10-8M at 25°C). Points were done in duplicate.

Table 4 shows the pKa of His64, change in pKa from the *B. lentus* wild type (WT) and the k_{cat}/K_M for seven modified N62C SBL mutants.

Table 4

SBL Enzyme	pKa of His64	ΔpKa	K_{cat}/K_M (s^{-1} mM^{-1})
WT	6.91	-	87
N62C	6.7	0.21	49
N62C-SMe	6.7	0.21	66
N62C-$SCH_2CH_2NH_3^+$	6.62.	0.29	52
N62C-$SCH_2CH_2SO_3^-$	7	0.09	86
N62C-Scyclohexyl	6.4	0.51	281
N62C-SBn	6.71	0.20	189
N62C-Sdecyl	6.19	0.72	197

As shown in Table 4, a very dramatic 0.5 unit decrease in the pKa of His64 is observed for the N62C-Scyclohexyl modified SBL as compared to the wild type. As such, it is possible to engineer altered pH profiles without altering surface charge.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as can be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

All publications and patents or applications referred to in the above specification are hereby incorporated by reference.

We Claim

1. A modified enzyme wherein one or more amino acid residues are replaced by cysteine residues, wherein the cysteine residues are modified by replacing the thiol hydrogen with a substituent group providing a thiol side chain selected from the group consisting of :

a) $-SR^1R^2$, wherein R^1 is an alkyl and R^2 is a charged or polar moiety;

b) $-SR^3$, wherein R^3 is a substituted or unsubstituted phenyl;

c) $-SR^4$, wherein R^4 is substituted or unsubstituted cyclohexyl; and

d) $-SR^5$, wherein R^5 is C_{10}-C_{15} alkyl.

2. The modified enzyme of Claim 1 wherein R^1 is C_{1-10} alkyl.

3. The modified enzyme of Claim 1 wherein R^2 is positively charged.

4. The modified enzyme of Claim 3 wherein R^2 is NH_3^+.

5. The modified enzyme of Claim 1 wherein R^2 is negatively charged.

6. The modified enzyme of Claim 5 wherein R^2 is SO_3^-.

7. The modified enzyme of Claim 1 wherein the enzyme is a protease.

8. The method of Claim 7 wherein the protease is a *Bacillus lentus* subtilisin.

9. The modified protease of Claim 7 wherein the amino acid replaced with a cysteine is an amino acid selected from the group consisting of asparagine, leucine, methionine and serine.

10. The modified protease of Claim 9 wherein the asparagine is in a subsite of the protease.

11. The modified protease of Claim 10 wherein the subsite is S_2.

12. The modified protease of Claim 11 wherein the asparagine is at position 62.

13. The modified enzyme of Claim 1 further comprising an alkyl group, R, before R^3 or R^4 to form $-SRR^3$ or $-SRR^4$.

14. The modified enzyme of Claim 13 wherein R is C_{1-10} alkyl.

15. A modified enzyme wherein one or more amino acid residues are replaced by cysteine residues, wherein the cysteine residues are modified by replacing the thiol hydrogen of the cysteine residue with a substituent group providing a thiol side chain $-SR^6$, wherein R^6 is a C_{1-6} alkyl, and wherein the amino acid residue is selected from the group consisting of asparagine, leucine and serine.

16. A method of producing a modified enzyme comprising:
   (a) providing an enzyme wherein one or more amino acids have been replaced with cysteine residues; and
   (b) replacing the thiol hydrogen with a substituent group providing a thiol side chain selected from the group consisting of :
      (i) $-SR^1R^2$, wherein R^1 is an alkyl and R^2 is a charged or polar moiety;
      (ii) $-SR^3$, wherein R^3 is a substituted or unsubstituted phenyl;
      (iii) $-SR^4$, wherein R^4 is substituted or unsubstituted cyclohexyl; and
      (iv) $-SR^5$, wherein R^5 is C_{10}-C_{15} alkyl.

17. The method of Claim 16 wherein R^1 is C_{1-10} alkyl.

18. The method of Claim 16 wherein R^2 is positively charged.

19. The method of Claim 18 wherein R^2 is NH_3^+.

20. The method of Claim 16 wherein R^2 is negatively charged.

21. The method of Claim 20 wherein R^2 is SO_3^-.

22. The method of Claim 16 wherein the enzyme is a protease.

23. The method of Claim 22 wherein the protease is a *Bacillus lentus* subtilisin.

24. The method of Claim 16 wherein the amino acid replaced with a cysteine is an amino acid selected from the group consisting of asparagine, leucine, methionine and serine.

25. The method of Claim 23 wherein the asparagine is in a subsite of the protease.

26. The method of Claim 25 wherein the subsite is S_2.

27. The method of Claim 26 wherein the asparagine is at position 62.

28. The method of Claim 16 further comprising an alkyl group, R , before R^3 or R^4 to form $-SRR^3$ or $-SRR^4$.

29. The method of Claim 28 wherein R is C_{1-10} alkyl.

30. A method of producing a modified enzyme comprising:
(a) providing an enzyme wherein one ore more amino acids have been replaced with cysteine residues and wherein the amino acids are selected from the group consisting of asparagine, leucine and serine; and
(b) replacing the thiol hydrogen of the cysteine residue with a substituent group providing a thiol side chain $-SR^6$, wherein R^6 is a C_{1-6} alkyl.

31. A detergent additive comprising the modified enzyme of Claims 1 or 15.

32. A feed additive comprising the modified enzyme of Claims 1 or 15.

33. A composition for the treatment of a textile comprising the modified enzyme of Claims 1 or 15.

34. The modified enzyme of Claim 1 wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is N62 and the thiol side chain is $-SR^1R^2$ wherein R^1 is CH_2CH_2 and R^2 is SO_3^-.

35. The modified enzyme of Claim 1, wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is N62 and the thiol side chain is $-SRR^3$ wherein R is CH_2 and R^3 is C_6H_5.

36. The modified enzyme of Claim 1, wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is N62 and the thiol side chain is $-SR^1R^4$ wherein R is CH_2 and R^4 is c- C_6H_{11}.

37. The modified enzyme of Claim 1, wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is N62 and the thiol side chain is $-SR^5$ wherein R^5 is n-$C_{10}H_{21}$.

38. The modified enzyme of Claim 1, wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is L217 and the thiol side chain is $-SR^5$ wherein R^5 is n-$C_{10}H_{21}$.

39. The modified enzyme of Claim 15, wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is N62 and the thiol side chain is $-SR^6$ wherein R^6 is $CH_2C(CH_3)_3$.

40. The modified enzyme of Claim 15, wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is N62 and the thiol side chain is $-SR^6$ wherein R^6 is C_5H_{11}.

41. The modified enzyme of Claim 15, wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is L217 and the thiol side chain is $-SR^6$ wherein R^6 is $CH_2C(CH_3)_3$.

42. The modified enzyme of Claim 15, wherein the enzyme is a *Bacillus lentus* subtilisin, the amino acid is L217 and the thiol side chain is $-SR^6$ wherein R^6 is C_5H_{11}.

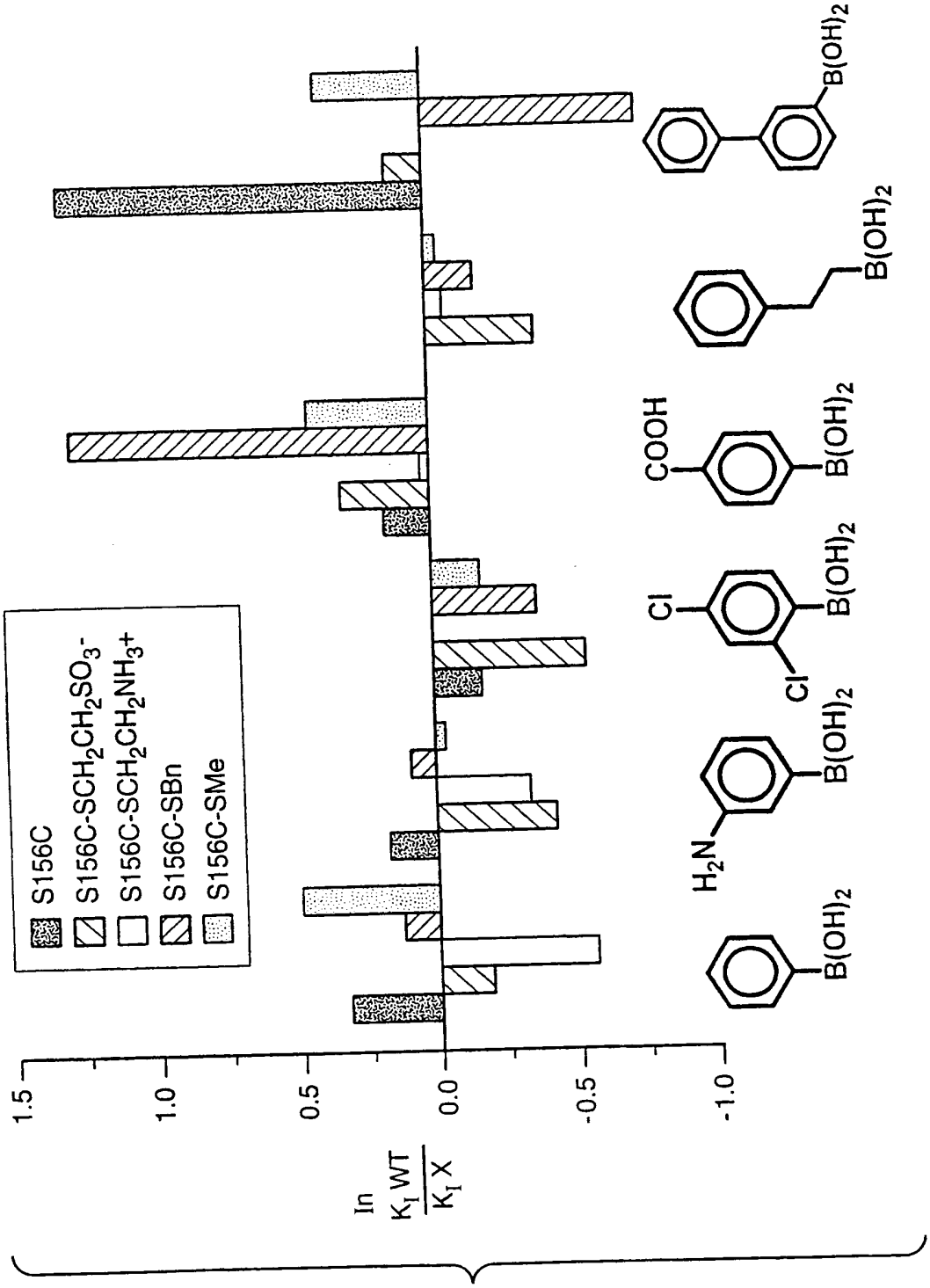

FIG._1

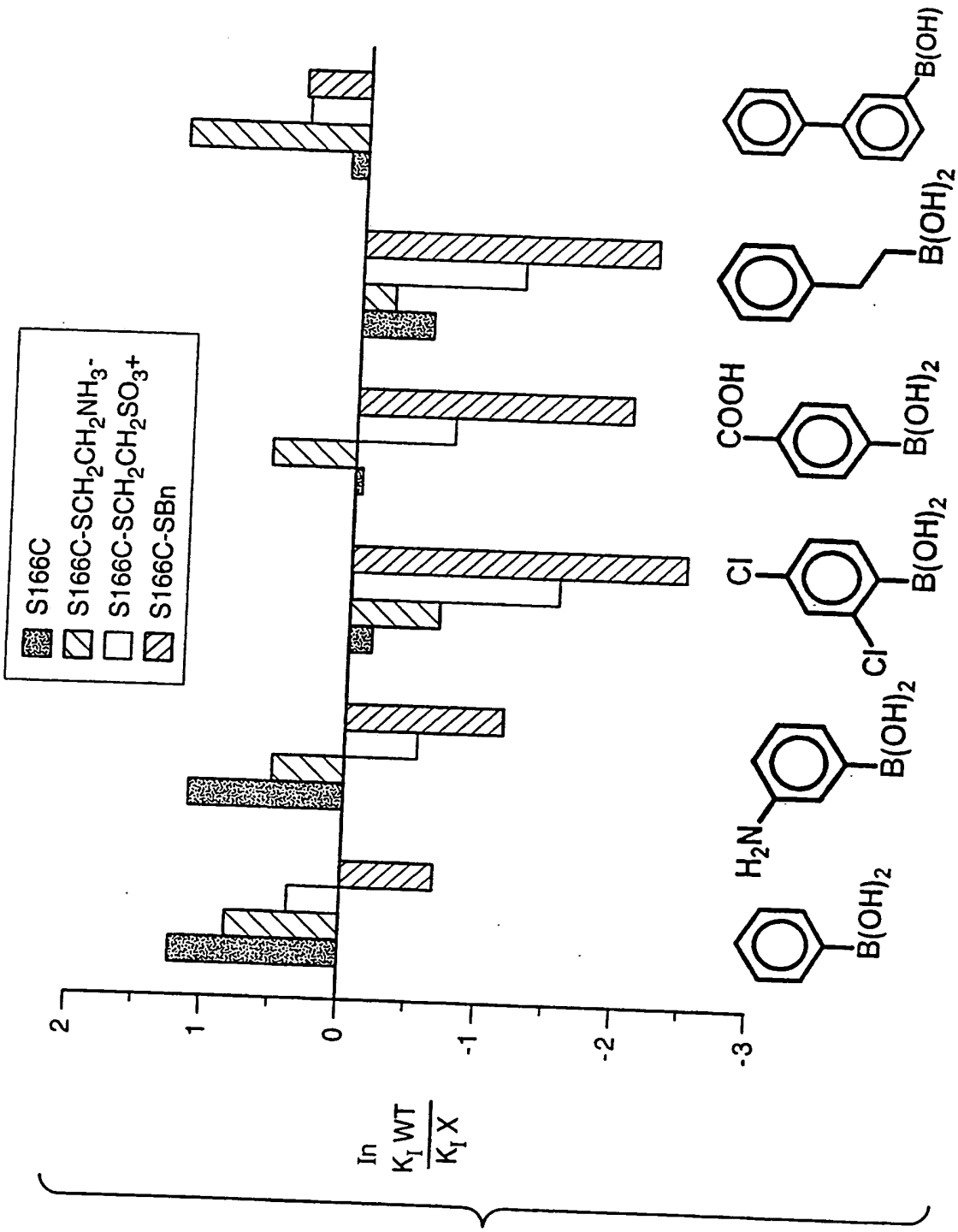

FIG._2

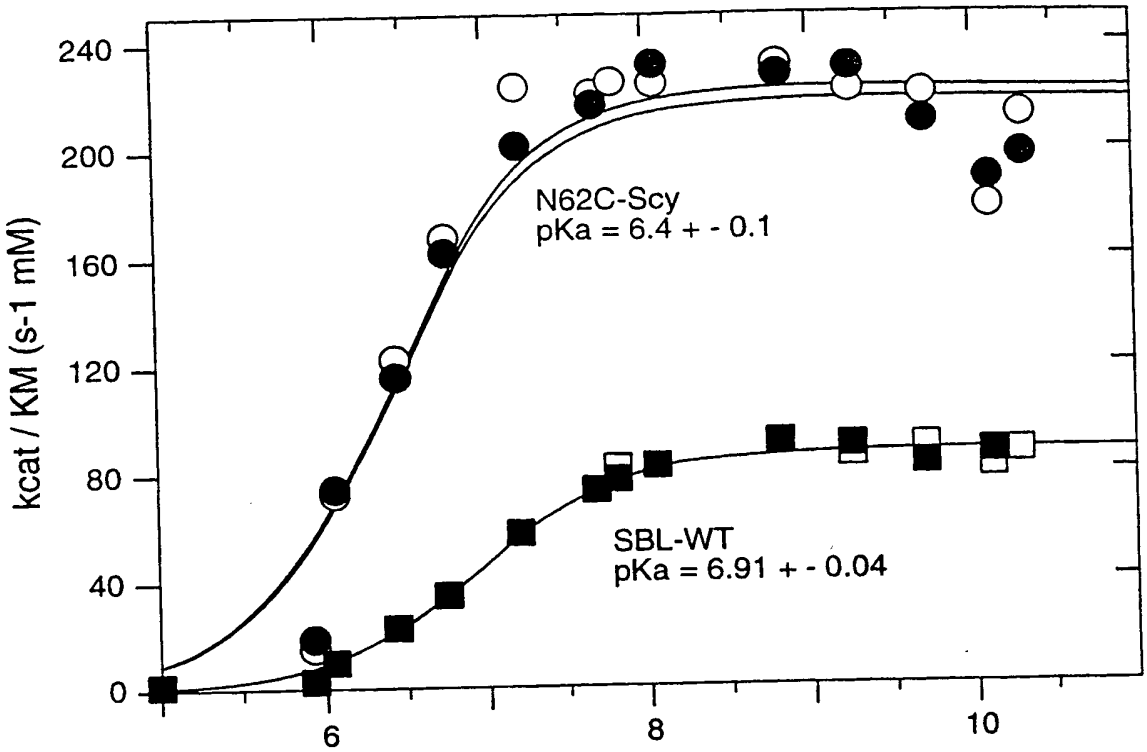

FIG._3

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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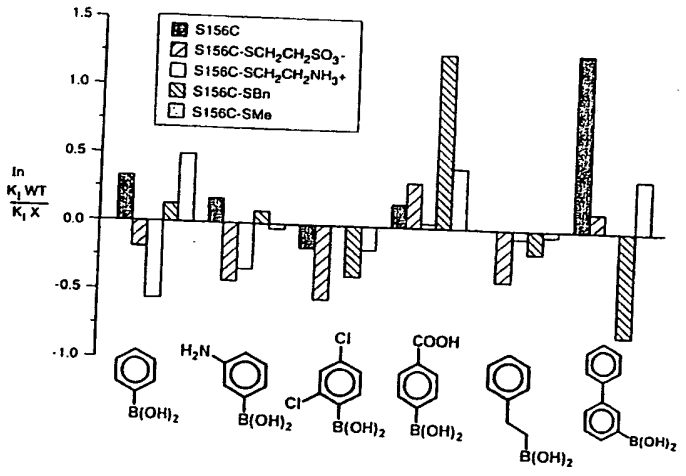
(54) Title: CHEMICALLY MODIFIED ENZYMES



(57) Abstract

Modified enzymes are provided in which at least one amino acid, such as asparagine, leucine, methionine or serine, of an enzyme is replaced with a cysteine and the thiol hydrogen is replaced with a substituent group providing a thiol side chain selected from the group consisting of: a) $-SR^1R^2$, wherein R^1 is an alkyl and R^2 is a charged or polar moiety; b) $-SR^3$, wherein R^3 is a substituted or unsubstituted phenyl; c) $-SR^4$, wherein R^4 is substituted or unsubstituted cyclohexyl; d) $-SR^5$, wherein R^5 is C_{10}–C_{15} alkyl; and e) $-SR^6$ wherein R^6 is a C_{1-6} alkyl. Also, methods of producing the modified enzymes are provided, as well as detergent and feed additives and a composition for the treatment of a textile. A method for using the modified enzymes in organic synthesis is additionally provided. Further, modified enzymes having improved activity, altered pH profile and/or wash performance are provided.

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INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 97/21446

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/00 C12N9/54 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BERGLUND, PER ET AL: "Altering the specificity of subtilisin B. lentus by combining site-directed mutagenesis and chemical modification" BIOORG. MED. CHEM. LETT. (1996), 6(21), 2507-2512 CODEN: BMCLE8;ISSN: 0960-894X, 6 November 1996, XP002063093	1-9, 15-24
Y	see the whole document	1,10-16, 25-42
Y	WO 91 16423 A (NOVONORDISK AS) 31 October 1991 cited in the application see the whole document	1,15,16, 30-33, 37-42

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
5 August 1998	28.08.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

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INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 97/21446

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BECH L AND BREDDAM K: "Chemical modification of a cysteinyl residue introduced in the binding site of carboxypeptidase Y by site-directed mutagenesis" CARLSBERG RESEARCH COMMUNICATIONS, vol. 53, 1988, pages 381-393, XP002063095	1-4,7,9, 13,14, 16-19, 22,24, 28,29
Y	see the whole document	1,13-15, 28-30, 34-36

Y	WYNN R AND RICHARDS F: "Chemical modification of protein thiols: formation of mixed disulfides" METHODS IN ENZYMOLOGY, vol. 251, 1995, pages 351-356, XP002063094 see the whole document	1,15,16, 30

Y	WO 96 27671 A (GENENTECH INC) 12 September 1996	10-12, 25-27, 31-37, 39,40
	see the whole document	

Y	EP 0 328 229 A (GIST BROCADES NV) 16 August 1989 see the whole document	31-33, 38,41,42

Y	BONNEAU P ET AL: "Alteration of the specificity of subtilisin BPN' by site directed mutagenesis..." JOURNAL OF THE AMERICAN CHEMICAL SOCIETY., vol. 113, 1991, pages 1026-1030, XP002073658 DC US cited in the application see the whole document	38,41,42

Y	GLOSS L AND KIRSCH J: "Examining the structural and chemical flexibility of the active site base, Lys-258, of Escherichia coli aspartate aminotransferase by replacement with unnatural amino acids" BIOCHEMISTRY, vol. 34, 1995, pages 12323-12332, XP002063096 see abstract	1,15,16, 30

Y	WYNN R ET AL: "Mobile unnatural amino acid side chains in the core of staphylococcal nuclease" PROTEIN SCIENCE, vol. 5, June 1996, pages 1026-1031, XP002073659 see the whole document	1,15,16, 30

	-/--	

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INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 97/21446

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BERGLUND, PER ET AL: "Chemical modification of cysteine mutants of subtilisin Bacillus lentus can create better catalysts than the wild-type enzyme" J. AM. CHEM. SOC. (1997), 119(22), 5265-5266 CODEN: JACSAT;ISSN: 0002-7863, 4 June 1997, XP002063097 see the whole document	1-42

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INTERNATIONAL SEARCH REPORT

Inter. ...nal application No.
PCT/US 97/21446

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)..

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internati Application No
PCT/US 97/21446

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9116423 A	31-10-1991	EP 0528864 A	03-03-1993
		FI 924698 A	16-10-1992
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		US 5324653 A	28-06-1994

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 2-6, 17-21 34 completely, 1, 7-12 16 22-27 31-33 partially

Modified enzyme wherein one or more amino acid residues are replaced by cysteine residues, wherin the cysteine residues are modified by replacing the thiol hydrogen with a substituent group of the general formula -SR1R2 in which R1 is an alkyl and R2 is a charged or polar moiety.

2. Claims: 35 completely, 1 7-14 16 22-29 31-33 partially

Modified enzyme wherein one or more amino acid residues are replaced by cysteine residues, wherin the cysteine residues are modified by replacing the thiol hydrogen with a substituent group of the general formula -SR3 in which R3 is a substituted or unsubstituted phenyl

3. Claims: 36 completely 1 7-14 16 22-29 31-33 partially

Modified enzyme wherein one or more amino acid residues are replaced by cysteine residues, wherin the cysteine residues are modified by replacing the thiol hydrogen with a substituent group of the general formula -SR4 in which R4 is substituted or unsubstituted cyclohexyl

4. Claims: 37 38 completely 1 7-12 16 22-27 31-33 partially

Modified enzyme wherein one or more amino acid residues are replaced by cysteine residues, wherin the cysteine residues are modified by replacing the thiol hydrogen with a substituent group of the general formula -SR5 in which R5 is C10-15 alkyl

5. Claims: 15 30 39-42 completely 31-33 partially

Modified enzyme wherein one or more amino acid residues selected from asparagine, leucine or serine are replaced by cysteine residues, wherin the cysteine residues are modified by replacing the thiol hydrogen with a substituent group of the general formula -SR6 in which R6 is C1-6 alkyl.

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